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Endogenous formaldehyde as a potential factor of vulnerability of atherosclerosis: involvement of semicarbazide-sensitive amine oxidase-mediated methylamine turnover

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Abstract

The mouse is known to be highly resistant to atherosclerosis. However, some inbred mouse strains are vulnerable to atherosclerosis when they are fed a high-cholesterol, high-fat diet. Increased deamination of methylamine (MA) and the subsequent production of formaldehyde has been recently shown to be a potential risk factor of atherosclerosis. In the present study semicarbazide-sensitive amine oxidase (SSAO)-mediated MA turnover in C57BL/6 mouse, a strain very susceptible to atherosclerosis, has been assessed in comparison to a moderate, i.e. BALB/c, and resistant, i.e. CD1, mouse strains. Kidney and aorta SSAO activities were found to be significantly increased in C57BL/6 in comparison to BALB/c and CD1 mice. A significant increase of urinary MA and formaldehyde were detected in C57BL/6. [^{14}C]MA following intravenous injection would be quickly metabolized by SSAO. The labeled formaldehyde product would cross link with proteins. C57BL/6 exhibits significantly higher labeled protein adducts than BALB/c and CD1 in response to [^{14}C]MA. The results indicated that mice vulnerable to atherosclerosis possess an increased SSAO-mediated MA turnover. The increase of production of formaldehyde, possibly other aldehydes, may induce endothelial injury or be chronically involved in protein cross-linking and subsequent angiopathy.

Keywords: Acetaldehyde; Amine oxidase; Formaldehyde; Methylamine; Methylglyoxal; SSAO

1. Introduction

Atherosclerosis is a complex process in which the lumen of the blood vessel becomes narrowed by cellular and extracellular substances to the point of obstruction. Atherogenesis includes initial injury, followed by fatty streak lesion, fibrous plaque formation, and finally advanced thrombosis. Hypertension and hyperglycemia are well-known risk factors for atherosclerosis and coronary heart disease [1,2]. Repeated endothelial injury has been suggested to be a potential initiating event in the development of atherosclerosis plaques [3,4]. Enhancement of proliferation and migration of vascular smooth muscle cells would be induced following endothelial injury and this is closely related to the genesis of atheroma. Experimentally, a strong association of atherosclerosis with serum levels of low density lipoproteins, i.e. lipoprotein (a), was observed [5,6]. Toxic oxygen products (free radicals) released from the activated neutrophils have been proposed to initiate endothelial damage [7]. Hyperglycemia plays a role in the initiation of endothelial injury in diabetes [8]. However, the factors for the initiation of endothelial injury are not completely understood. Experimental approaches rely heavily on in vitro systems, which could be misleading. Until recently, atherogenesis studies

Abbreviations: SSAO, semicarbazide-sensitive amine oxidase (EC 1.4.3.6); MA, methylamine; DNPH, 2,4-dinitrophenylhydrazine; MDL-72974A, (E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride.

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were mainly in primates and rabbits. Unfortunately, these systems cannot provide a sufficiently large number of animals. Rodents are unfortunately resistant to atherosclerosis and therefore are not considered to be a good model for the study of atherosclerosis [9]. However, certain mouse strains, such as C57BL/6, after a long term high cholesterol and fat diet, would develop atherosclerosis particularly near the aortic valve leaflets, whereas other inbred strains, i.e. C3H/HeJ, would not [10]. It has been shown that mouse genes, such as ApoE or LDL receptor, are related to atherogenesis in mutant inbred strains.

Recently, it was proposed that increased semicarbazide-sensitive amine oxidase (SSAO) activity may be involved in endothelial damage, protein crosslinkage and atherogenesis [11,12]. Methylamine (MA) has been found to be deaminated by SSAO [13,14] and toxic formaldehyde and hydrogen peroxide [15,16] are formed. SSAO resides predominantly in the plasma membrane of vascular smooth muscle cells, such as blood vessels [17–19], retina and brain microvessels [20] and circulating in the blood [21]. In the present study we compared aorta and kidney SSAO activities, urinary excretion of MA and formaldehyde in vulnerable and resistant mice strains with respect to atherosclerosis. The effect of SSAO inhibitors on the metabolism of MA was also examined. Our results are consistent with the idea that increased deamination of MA may be involved in atherogenesis in vulnerable C57BL/6 mice.

2. Materials and methods

2.1. Materials

[7-^{14}C] Benzylamine hydrochloride (59 mCi/mmol) was purchased from Amersham Life Science (Amersham International, Buckinghamshire, UK). (E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride (MDL-72974A) was kindly provided by Marion–Merrell–Dow (Cincinnati, OH). All other chemicals are of analytical grade.

2.2. Animal experiments

Male C57BL/6 mice weighing 20 g were obtained from Charles River, St. Constant, Canada. Male CD1 Swiss white and BALB/c mice were supplied from Animal Resource Centre, University of Saskatchewan. The animal studies were in strict accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the University of Saskatchewan Animal Care Committee. The mice were housed in hanging wire cages with free access to food and water on a 12 h light/dark cycle (lights on 6:00 h) at a temperature of 19–20°C.

2.3. Urine collection

Mice were placed in metabolic cages (Nalgene, Rochester, NY) for urine collection. The urine collecting vessels were positioned over a Styrofoam containers filled with dry ice which permitted the collection of urine in a frozen state over the collection period. The animals were allowed free access to tap water. During urine collection food was withheld.

2.4. Determination of SSAO activity

SSAO activity was determined by a radio-enzymatic procedure using ^{14}C-labeled benzylamine as substrate following our previously described procedure [14]. The SSAO enzyme preparations were pre-incubated with clorgyline (1×10^{-4} M) at room temperature for 20 min to ensure that any monoamine oxidase activity, if present, was completely inactivated. The enzyme was then incubated in the presence of benzylamine (5×10^{-5} M, 0.1 μCi) in a final volume of 200 μl at 37°C for 30 min. The enzyme reaction was terminated by adding 200 μl 2 M citric acid. The oxidized products were extracted into 1 ml toluene:ethyl acetate (1:1, v/v), of which 600 μl was then transferred to a counting vial containing 10 ml Omnifluor cocktail (New England Nuclear, Boston, MA). Radioactivity was assessed by liquid scintillation counting (Beckman LS-7500, Fullerton, CA). One unit of enzyme activity is defined as 1 nmol of product formed per min per mg of protein.

2.5. Determination of MA

Urinary MA was determined using an HPLC-fluorometric procedure as previously described [22]. Briefly, internal standard isopropylamine (4 μg in 100 μl) was added to the 250 μl urine samples and made to 2 ml with nanopure water. The samples were applied to a small column of CG-50 amberlite (0.5×2.5 cm), rinsed with 10 ml water and the amines were then eluted with 2 ml 1 N HCl. The sample eluates (100 μl) were derivatized with *o*-phthaldialdehyde (OPA) (100 μl) under alkaline condition (pH 10.4), and 50 μl of the derivatized samples elutes were separated using a Shimadzu HPLC system (Sil-9A auto injector) equipped with a pre-column derivatization program. The fluorescent amine derivatives were separated in an analytical Ultrasphere I.P. column packed with octadecyl-bonded spherical-5 μm silica particles (250×4.6 mm I.D.) (Beckman, Toronto, Ontario). The column was eluted with 65% methanol at a flow rate of 1.0 ml/min using a SSI 222B solvent delivery system (State College, PA). For quantitative assessment, a programmable fluorescence detector (Hewlett Packard, HP1046A) with excitation at 360 nm and emission at 445 nm was employed. The peak areas were integrated using a

Spectra-Physics SP-4290 integrator. The peak ratios of MA/isopropylamine were used for the calculation of urine levels of MA.

2.6. Determination of urinary aldehydes

A modified HPLC/spectrophotometric method [23] has been applied for the determination of aldehydes. Aldehydes are derivatized with 2,4-dinitrophenyl-hydrazine (DNPH). Propionaldehyde was used as an internal standard. The DNPH reagent was prepared at the concentration of 10 mM in distilled water containing 50% acetonitrile. In 25 ml screw-capped tubes, 3.4 ml of distilled water, 0.8 ml of 50 mM phosphate buffer, 0.5 ml aliquots of urine, 0.1 ml of 10 μM internal standard solution and 0.2 ml of the DNPH reagent were mixed, and followed by the incubation at 37°C for 10 min. The hydrazone products were vigorously extracted twice with 10 ml of pentane. The pentane extracts were evaporated to dryness at 45°C in water bath and the precipitates were dissolved in 400 μl solvent, i.e. the solution of the mobile phase for HPLC. Aliquots of the concentrated samples were subject to HPLC analysis.

HPLC system composed of a SSI 222B HPLC pump (Milford, MA), a WISP 712B Autoinjector (Waters, Millipore, Milford, MA) and a Spectra-Physics recorder (San Jose, CA). The separation was performed using a reversed-phase Ultrasphere I.P. analytical column (250 × 4.6 mm I.D., Beckman, Toronto, Ontario). Elution was isocratic with 20 mM phosphate buffer, pH 4.6, containing 32% acetonitrile and 8% 2-methylpropanol at a flow rate of 1.0 ml/min. Spectrophotometric detection was conducted using a Lambda-Max Model 481 LC spectrophotometer (Waters, Millipore, Milford, MA) at 330 nm. Data represent the average of four or more analyses.

2.7. Determination of residual radioactivity following administration of [^{14}C]-MA

Radioactively labeled MA (10 μCi, 100 nmol) was administered to the mice via tail intravenous injection. After 120 hr the mice were killed and different tissues were dissected. Aliquots of these tissues were homogenized in 0.05 M phosphate buffer (pH 7.2) (1:3, w/v) and aliquots of the homogenates were transferred to counting vials containing 0.5 ml Solvable and 10 ml ACS scintillation fluid (Amersham, Oakville, Ontario). Radioactivities were assessed by liquid scintillation spectrometry (Beckman LS-7500).

2.8. Statistic

The results will be assessed using analysis of variance (ANOVA). In general, the null hypothesis used for all analyses will be that the factor has no influence on the measured variable and significance will be accepted at the $>95\%$ confidence level.

3. Results

3.1. Comparison of aorta and kidney SSAO activities in different mouse strains

Fig. 1 shows the specific activities of SSAO in aorta and kidney from C57BL/6, BALB/c and CD1 Swiss white mice. SSAO activity was found to be significantly higher in C57BL/6 mice, followed by BALB/c and with the lowest activity in CD1 mice. Nearly 5-fold increase of SSAO activity in kidney from C57BL/6 in comparison to CD1 mice was detected.

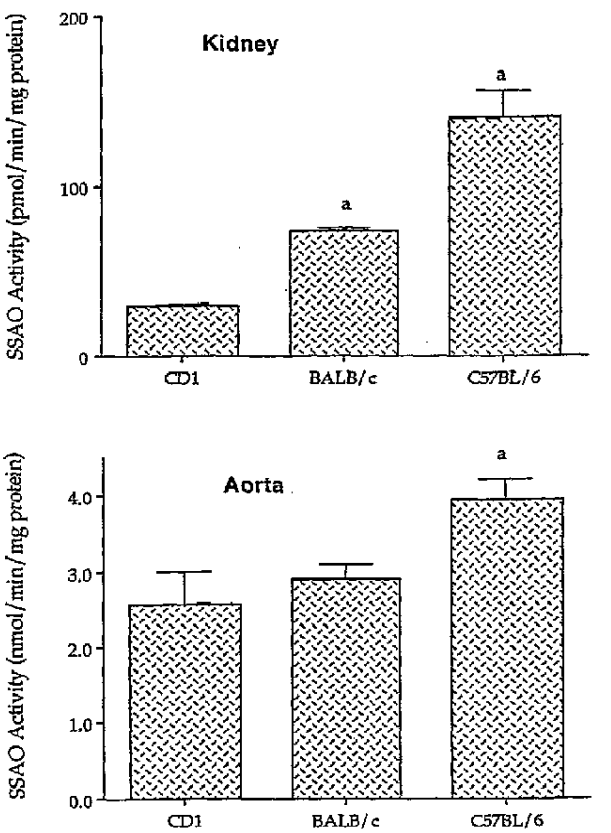

Fig. 1. Aorta and kidney semicarbazide-sensitive amine oxidase (EC 1.4.3.6) (SSAO) activities in different mouse strains. SSAO activity was determined using tissue homogenates pretreated with clorgyline (1×10^{-4} M) (to ensure no monoamine oxidase activity). [^{14}C]Benzylamine (4×10^{-4} M) was used as substrate. Each value is the mean ± S.E. of four independent experiments. [a] $P < 0.01$ in comparison to CD1 mouse.

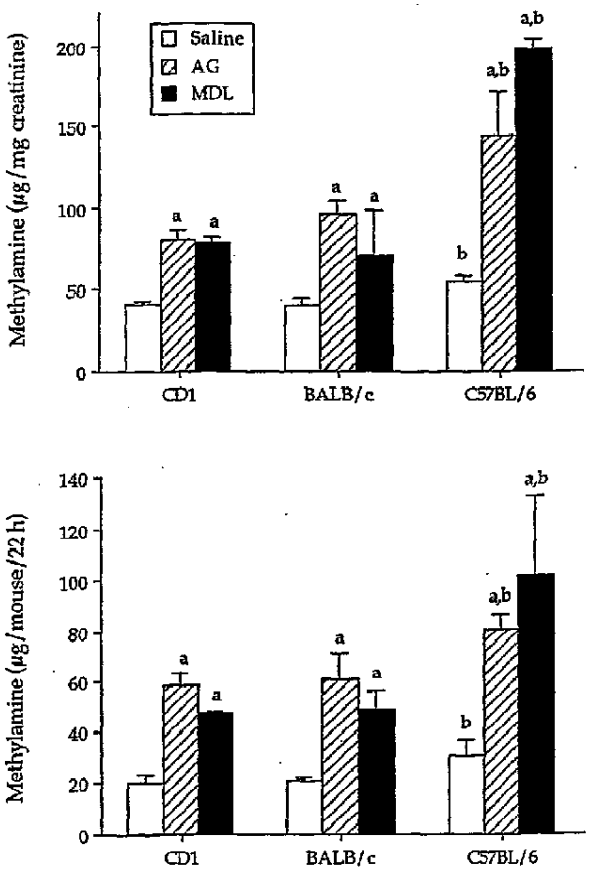

Fig. 2. Effect of SSAO inhibitors on urinary methylamine (MA) excretion in different mouse strains. Aminoguanidine (25 mg/kg), MDL-72974A (2 mg/kg) and saline were administered intraperitoneally to mice and then 22 h urine was collected. MA was assessed by an HPLC-fluorometric procedure as described in Section 2. Upper panel shows MA levels based on creatinine and lower panels indicates the total amount of MA excretion. Creatinine was determined using the picric acid method. Values are mean ± S.E. of three animals at each dose; [a] $P < 0.01$ in comparison to corresponding untreated groups; [b] $P < 0.01$ in comparison to corresponding CD1 groups.

3.2. Urinary levels of MA and the effect of SSAO inhibitors

The urinary excretion of MA from the three mouse strains were assessed by a fluorometric HPLC procedure. MA levels were assessed based on either urinary creatinine or on total excretion per mouse. As can be seen in Fig. 2, MA level in the saline control C57BL/6 mouse is significantly higher than those in the CD1 Swiss mouse. SSAO inhibitors, aminoguanidine [24] and MDL-72974 [25], increase the urinary MA levels in all strains of mice. However, a larger extent of increase of urinary MA was observed in the C57BL/6 mouse. Following a single injection of MDL-72974A (2 mg/kg, i.p.) (Fig. 2, upper panel) approximately 3-fold MA excretion was detected in untreated C57BL/6 than in CD1 mouse. Urinary MA level after SSAO inhibition in C57BL/6 was also significantly higher than in BALB/c mouse.

3.3. Urinary aldehydes

Formaldehyde levels based on either creatinine or total excretion per mouse over a 22 h collection period are illustrated in Fig. 3. A significantly higher urinary formaldehyde level was detected in the C57BL/6 mouse, and followed by BALB/c and CD1 strains.

3.4. Formation of formaldehyde-protein adducts

Following administration of [^{14}C]MA, a significant amount of long-lasting residual radioactivity was detected in different mouse tissues [12]. The residues were found to be primarily formaldehyde-protein adducts. This experimental approach has been applied to assess the in vivo deamination of MA by SSAO and as a marker of the interaction of SSAO-mediated formaldehyde with biological constituents [12,26]. Fig. 4 shows the distribution of the residual radioactivities in various tissues from different mouse strains. Formaldehyde-protein residue formation was observed to be significantly increased in kidney, lung and liver in C57BL/6 mice.

4. Discussion

SSAO is an intriguing enzyme, but receives very little attention. It catalyzes the deamination of aliphatic

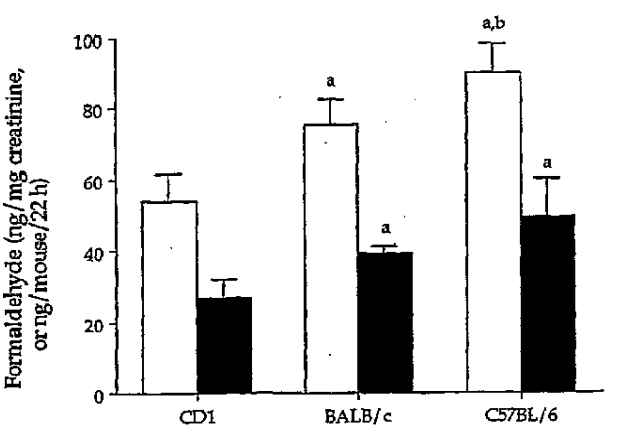

Fig. 3. Urinary formaldehyde levels in different mouse strains. Formaldehyde was determined by an HPLC-spectrophotometric procedure as described in Section 2. Formaldehyde levels based on creatinine (open bar) or the total amount of formaldehyde excretion (filled bar) are indicated. Creatinine was determined using the picric acid method. Values are mean ± S.E. of three animals at each dose; [a] $P < 0.01$ in comparison to corresponding CD1 group; [b] $P < 0.05$ in comparison to corresponding BALB/c group.

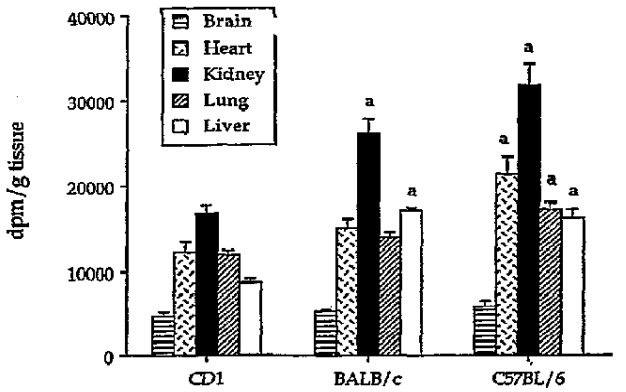

Fig. 4. Residual radioactivity in different tissues following administration of [^{14}C]methylamine (MA) in different mouse strains. The mice were injected with [^{14}C]MA (10 μCi, 170 pmol/mouse, via intravenous injection). The residual radioactivity in different tissues as indicated in the figures was assessed 120 h after administration of the labeled MA. The overall recovery of the residual radioactivities were approximately 10%. Values are means ± S.E. of the mean of four animals.

amines, such as MA [13–16], aminoacetone [27] and allylamine [28] and toxic aldehydes, namely, formaldehyde, methylglyoxal and acrolein are produced, respectively.

$$CH_3NH_2 + O_2 + H_2O \xrightarrow{SSAO} HCHO + H_2O_2 + NH_3$$

$$CH_3COCH_2NH_2 + O_2 + H_2O \rightarrow CH_3COCHO + H_2O_2 + NH_3$$

$$CH_2{=}CHCH_2NH_2 + O_2 + H_2O \rightarrow CH_2{=}CHCHO + H_2O_2 + NH_3$$

Allylamine, an industrial chemical, has been found to cause extensive and progressive vascular and myocardial lesions in several mammalian species [29]. The vascular damage induced in this model exhibits features (e.g. intimal smooth muscle cell proliferation) very similar to those seen in human atherosclerosis. The SSAO inhibitor semicarbazide can protect against the progress of damage caused by allylamine [28]. The vascular cytotoxicity is due to the selective location of SSAO in these tissues [30]. Unlike allylamine, MA and aminoacetone are present endogenously [31]. Since both amines and SSAO are circulated in blood serum, it was proposed that toxic formaldehyde [11] and methylglyoxal [32] derived via SSAO-mediated deamination may potentially initiate endothelial damage and protein crosslinkage, and subsequently cause atherogenesis.

The C57BL/6 inbred mouse is well known to be vulnerable to atherosclerosis. Its SSAO activities were found to be significantly higher than the atherosclerotic resistant mouse strains, such as CD1. It seems to be interesting to know whether such a higher level of SSAO activity may lead to an increase of deamination of MA and thus generate harmful formaldehyde (or other aldehydes) in C57BL/6 mice. We assessed the urinary excretion of MA as well as formaldehyde in these mice. The baseline MA levels in C57BL/6 mice were found to be significantly higher (approximately 35% increase) than in the urine from BALB/c and CD1 mice. Such an increase of MA became dramatic (approximately two to 3-fold increase) when SSAO activities were blocked by SSAO inhibitors, i.e. MDL-72974A or aminoguanidine. The results indicate that not only is MA catabolism enhanced, but also the availability of MA is higher in the C57BL/6 mouse. The increase of SSAO activity could be a result of up-regulation in response to the presence of high amount of MA.

The detection of high levels of formaldehyde excretion in C57BL/6 mouse indicated that turnover of MA is also increased. This conclusion is further supported by examining the protein adduct formation following administration of [^{14}C]MA. This experimental approach was previously established, namely, [^{14}C]MA would be deaminated and [^{14}C]formaldehyde would cross-link with proteins [12]. SSAO inhibitor can effectively block such adduct formation. Following administration of [^{14}C]MA C57BL/6 mice exhibited significantly higher labeled protein complexes in different tissues in comparison to BALB/c and CD1 mice. The results show that SSAO-mediated MA turnover is highest in C57BL/6, followed by BALB/c and CD1. This is consistent with the vulnerability of contracting atherosclerosis in these mice.

Formaldehyde is extremely reactive. It is capable of inducing intra- and inter-molecular cross-linkage between proteins and between proteins and DNAs. The cross-linkage of long lasting proteins, such as in the basal membranes of blood vessels, may cause harmful consequences. Formaldehyde has also been shown to enhance protein glycation [31]. It is perhaps also interesting to note that the C57BL/6 mouse is also susceptible to azocasein-induced amyloidosis [33], in which advanced protein crosslinkage is involved. Perhaps the SSAO-mediated formation of aldehydes may be involved in the amyloidogenesis and plaque formation.

Serum SSAO activity has been found to be increased in diabetic patients [34–36] and diabetic animals [37,38]. It is perhaps interesting to note that aortae SSAO activity was not increased in the diabetic rats [37] or atherosclerotic patients [39]. Atherosclerosis is closely linked with diabetic complications. It was proposed that formaldehyde derived from increased SSAO catalyzed deamination of MA may be related to vascular complications in diabetes [11]. Recently serum SSAO has been also shown to be significantly increased in patients with congestive heart failure [40]. Also MA has been shown to be 20-fold higher in uremia patients

than in control populations [41]. This has been found to be due to the accumulation of creatinine (a precursor of MA) and renal clearance dysfunction [22]. Nephropathy is the most common complication among diabetic patients. It is yet to be established, whether the increase of deamination of MA causes renal failure or whether the release of SSAO into the circulatory system is a result of renal failure.

In conclusion, an increase of SSAO-catalyzed MA turnover has been found to be associated with the vulnerability to atherosclerosis in mice. Increase of endogenous toxic formaldehyde could induce cross-linkage of proteins, cause hardening of blood vessels, initiate endothelial injury and subsequently produce atheroma in some pathological conditions. The results strongly support the hypothesis that SSAO-mediated generation of toxic aldehydes could be a risk factor for atherosclerosis.

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